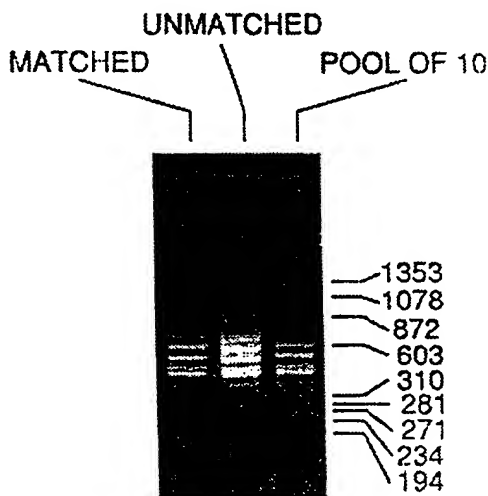




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<p>(21) International Application Number: PCT/US93/10722 (22) International Filing Date: 8 November 1993 (08.11.93) (30) Priority data: 07/974,447 12 November 1992 (12.11.92) US (71) Applicant: COLD SPRING HARBOR LABORATORY [US/US]; One Bungtown Road, Cold Spring Harbor, NY 11724 (US). (72) Inventors: WIGLER, Michael ; 1 Walden Court, Lloyd Harbor, NY 11743 (US). LISITSYN, Nikolai ; P.O. Box 100, Cold Spring Harbor, NY 11724 (US). (74) Agents: ROWLAND, Bertram, I. et al.; Flehr, Hohbach, Test, Albritton &amp; Herbert, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).</p>		<p>(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.</p>

(54) Title: A REPRESENTATIONAL APPROACH TO DNA ANALYSIS



(57) Abstract

Methodology is provided for developing probes for identifying sequence differences between two related DNA populations, sets of DNA fragments or collections of restriction-endonuclease-cleaved DNA or cDNA. The method employs an initial stage to obtain a representation of both DNA populations, namely using the PCR to produce relatively short fragments, referred to as amplicons. Tester amplicons containing target DNA, sequences of interest, are ligated to adaptors and mixed with excess driver amplicons under melting and annealing conditions, followed by PCR amplification. The process may be repeated so as to greatly enrich the target DNA. Optionally, the target DNA may then be cloned and the DNA used as probes.

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**A REPRESENTATIONAL APPROACH TO DNA ANALYSIS**  
**CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of application serial no. 07/974,447, filed November 12, 1993.

5 **INTRODUCTION**

**Technical Field**

The field of this invention is DNA analysis.

**Background**

Comparative genomic DNA analysis holds promise for the  
10 discovery of sequences which may provide for information concerning polymorphisms, infectious DNA based agents, lesions associated with disease, such as cancer, inherited dominant and recessive traits, and the like. By being able to detect particular DNA sequences which have a function or affect a  
15 function of cells, one can monitor pedigrees, so that in breeding animals one can follow the inheritance of particular sequences associated with desirable traits. In humans, there is substantial interest in forensic medicine, diagnostics and genotyping, and determining relationships between various  
20 individuals. There is, therefore, substantial interest in providing techniques which allow for the detection of common sequences between sources and sequences which differ between sources.

The mammalian genome is extraordinarily large, having about  
25  $6 \times 10^9$  bp. The human genome project has initiated an effort to map and sequence the entire genome. However, much of the early work will be directed more toward determining the site

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of particular genes, than determining contiguous sequences of a particular chromosome.

Because of the complexity of the human genome, there is a very substantial handling and processing problem with the human genomic DNA. In order to deal with such a large amount of DNA, one must develop processes which allow for simplification and selection, while still providing the desired information. Therefore, efforts must be made which will provide for opportunities which will allow to greater or lesser degrees, dissecting portions of a genome of interest, where comparisons can be made between two different sources of DNA.

#### Relevant Literature

Efforts at difference analysis at the level of the genome are described by Lamar and Palmer, *Cell* 37, 171 (1984); Kunkel et al., *Proc. Natl. Acad. Sci. USA* 82, 4778 (1985); Nussbaum et al., *Proc. Natl. Acad. Sci. USA* 84, 6521 (1987); Wieland et al., *Proc. Natl. Acad. Sci. USA* 87, 2720 (1990); Straus and Ausubel, *Proc. Natl. Acad. Sci. USA* 87, 1889 (1990).

#### SUMMARY OF THE INVENTION

Representational difference analysis is provided to determine similarities or differences between two related sources of DNA. In a first step, a representative portion of each genome is prepared, using a restriction endonuclease (RE1), ligation of partially double-stranded adaptors, and the polymerase chain reaction, and cleavage with RE1 to provide a population of relatively small DNA fragments referred to as "amplicons." This stage may be repeated in separate analyses with different restriction endonucleases or different schemes, e.g., fractionation.

The first amplicon of source DNA is referred to as the "driver," which amplicon is used in substantial excess in the subsequent processing of the other, "tester" amplicon. The tester includes the "target" DNA, which DNA is absent in or is present in reduced amounts in driver amplicon. Partially double-stranded PCR adaptors are ligated only to tester amplicon fragments, and the tester and driver DNA combined,

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melted and reannealed. The termini of the amplicons are filled in and using primers complementary to the adaptors, the DNA mixture is subjected to amplification, wherein the target DNA will undergo exponential amplification and be substantially enriched as compared to driver DNA and non-target tester DNA, which anneals to the driver DNA. Adaptors may then be removed and the cycle repeated using different adaptors. Various modifications may be employed at different stages to further enhance selection of the target DNA.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a gel electrophoresis and genomic blot analysis of the application of RDA to isolate probes that detect gene amplification;

Fig. 2 is a gel electrophoresis analysis of gene amplification using drivers from different sources;

Fig. 3 is a sequence comparison of difference product P35 from human prostate cancer with rat retrotransposon RatL1RnB6; and

Fig. 4 is a gel electrophoresis analysis of difference sequences between two cDNA populations.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for representational difference analysis ("RDA") between two sources of DNA. The method permits the detection of sequences which differ between the two sources, where under selective conditions of hybridization, DNA from one of the two sources is not significantly hybridized to DNA from the other source. Sources include genomes, sets of DNA fragments, usually  $\geq 0.2$  kbp, collections of restriction endonuclease-cleaved fragments, cDNA or cDNA libraries, etc. The method involves a first step, referred to as representation, and then two or more further steps referred to as subtractive and kinetic enrichment, which may be repeated in order to provide for substantial enrichment of the sequences of interest.

For the purpose of this invention, a number of coined terms will be used. "Driver" DNA is DNA from a source which will be

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used to determine the presence of DNA in a second source, the "tester" source. Those fragments that are unique or in higher concentration to the tester DNA, as compared to the driver DNA, will be referred to as "target" DNA. The DNA sequences are  
5 obtained in a first stage resulting from restriction endonuclease digestion, followed by linkage of adaptors and then amplification with primers complementary to the adaptors. The resulting DNAs are referred to as "amplicons." The amplicons will be characterized by being under about 2 kb and  
10 usually at least about 0.5 kb, where the termini will normally have the same restriction endonuclease recognition sequence prior to linkage to the adaptors.

The subject application may find use in a wide variety of situations. In determining the presence or absence of  
15 particular DNA sequences, particularly associated with recessive or dominant traits, one can compare two related sources of DNA to determine whether they share the particular sequence, where the sequence may be a coding or non-coding sequence, but will be inherited in association with the DNA  
20 sequence(s) associated with the trait. One can use the subject method in forensic medicine, to establish similarities between the DNA from two sources, where one is interested in the degree of relationship between the two sources. The subject method can also be applied in the study of diseases, where one can  
25 investigate the presence of a sequence associated with infection, such as a viral sequence which may or may not be integrated into the genome. One may also use the subject methodology in studying changes in the genome as a result of cancer, where cancerous cells may be compared to normal wild-  
30 type cells. Thus, the subject methodology has application for detecting genetic rearrangements, genetic loss, gene or other DNA amplification, for identification of DNA from pathogenic organisms integrated into the genome or present in the cellular host, for identification of polymorphisms located at or near  
35 genes associated with inherited disorders, for identification of genes which are expressed in a particular cellular host, identification of lesions in neoplastic cells, and the like.

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In carrying out the subject method, there are concerns which should be considered when applying the subject method. The PCR may be a source of artefacts, due to the stochastic nature of the process. Therefore, each candidate difference product  
5 should be tested for its presence or absence in tester and driver amplicons. Another source of artefact may occur during tissue sampling. Normal flora contaminating a specimen of tester will be readily enriched during difference analysis if that flora is not also present in driver. Genetic mosaicism  
10 may be encountered. In situations where one is dealing with polyclonal tissue, such as in a cancer biopsy, there must be a minimum proportion of cells which has the particular mutation in order to be able to detect the presence of the mutation. Therefore, it would be desirable to use cultures of cancer  
15 cells or highly purified cancer cells obtained by physical separation as the source for the tester DNA. In the case of discovery of pathogens, there should be a careful matching of the polymorphisms from the infected and uninfected DNA source. In the latter case, tester and/or driver DNA may derive from  
20 the same individual, come from an identical twin, come from separate but related individuals, be the pooled DNA from the parents of the tested individual, be pooled DNA from related sources, e.g. cell strains, common genetic dysfunction, or common trait, or the like.

25 Finally, not all restriction endonucleases will be equivalent in the ease with which target DNA may be identified. Therefore, in each case it will be desirable to use a plurality of restriction endonucleases in separate determinations, not only to ensure that one obtains target DNA within a reasonable  
30 number of cycles, but also to increase the number of target DNA sequences that may be obtained.

Turning now to the specific process, the first stage is the isolation of DNA. As already indicated, the DNA may be from any source, eukaryotic or prokaryotic, invertebrate or  
35 vertebrate, mammalian or non-mammalian, plant or other higher eukaryotic source. \*\*\*While, from the standpoint of direct application to human interests, the sources will be human DNA, the subject methodology is applicable to any complex genome,

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where one is interested in identifying the presence or absence of related DNA, such as laboratory animals, plants, domestic animals, or in any other situation where an inbred or outbred population is of interest. Normally, the DNAs will be from  
5 closely-related sources, so that the number of target DNA sequences which are obtained will be relatively restricted in number, frequently being fewer than about  $10^4$ , usually fewer than about  $10^3$ , different sequences. While genomic DNA will usually be the source of driver and tester DNA, cDNA may also  
10 be used, where one is interested in the differences between two cDNA populations from two different mRNA sources.\*\*\*

In the first stage, the DNA is isolated, freed of protein, and then substantially completely digested with a restriction endonuclease which provides for relatively infrequent cutting.  
15 Usually, the restriction endonuclease will have a consensus sequence of at least six nucleotides and may provide for blunt ends or staggered ends, usually staggered ends. Various restriction endonucleases may be employed, such as BamHI, BglII, HindIII, etc. After digestion of the DNA, double-  
20 stranded oligonucleotide adaptors are ligated to the ends of each of the strands of the DNA from the driver and the DNA from the tester. The adaptor will usually be staggered at both ends, with one strand being longer and serving as the sequence complementary to the primer. The adaptor will be double-  
25 stranded and have one end complementary to the ends of the dsDNA from the digestion. The DNA from the two sources is then separately amplified, by adding primer and using the polymerase chain reaction with extension for the last round, usually employing at least 10 cycles, more usually at least 15 cycles  
30 and generally not more than about 30 cycles, more usually not more than about 25 cycles and preferably about 20 cycles. After this number of cycles, for the most part, the fragments will be mainly less than about 2 kb, usually below about 1.0 kb. The adaptors are then removed by restriction  
35 endonuclease digestion and physical separation, using any convenient means.

As distinct from a physical fractionation, the amount of starting material is not limiting when using representation.



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When employing amplicons of mammalian DNA after cleavage with BamHI, BglII and HindIII, the estimated complexity of the resulting amplicons are 55-fold, 13-fold and 8-fold less than the complexity at the starting genomic DNA, respectively  
5 (Bishop et al., *Am. J. Hum. Genet.* 35, 795 [1983]).

Other methods of representing the genome to reduce its complexity may be employed. For example, cleavage with a more frequently cutting enzyme, e.g. a 4 nt consensus sequence restriction enzyme, followed by addition of adaptors, PCR  
10 amplification and size fractionation, will achieve this end. Another method might use oligonucleotides as primers to repetitive DNA in the genome to amplify a representational portion of the genome, flanking repetitive sequences.

In the next phase, subtractive and kinetic steps are employed  
15 in a single operation of hybridization and amplification. If desired, the steps may be separated, but will preferably be done contemporaneously. The first aspect of this stage is the ligation of PCR adaptors to the 5' ends of tester amplicon fragments or the products of previous rounds of enrichment,  
20 when the procedure is reiterated. Ligation to the 3' ends of tester amplicon is to be avoided, which can be achieved, for example, by using adaptors that are not phosphorylated at their 5' ends. Usually, the adaptor chain complementary to the primer will be at least about 12 nt, more usually at least 17 nt, and  
25 generally fewer than about 200 nt, more usually fewer than about 100 nt. Any convenient method for ligation of the adaptors to the 5' ends may be employed, as appropriate.

The tester amplicon fragments joined to the adaptors are then combined with the driver amplicon fragments and melted and  
30 allowed to reanneal. The driver amplicon fragments will be present in substantial excess, usually at least 5-fold excess, and the excess may exceed 50 or more, usually not exceeding about  $10^8$ -fold excess, more usually not exceeding 500-fold excess. The ratio of driver DNA to tester DNA need not be  
35 constant for the different rounds. Usually, the ratio will increase with successive rounds where the increase may vary from about 1:1 to  $10^3$ . The initial ratio will generally be in the range of about 10 to 1000-fold excess. Conveniently,

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melting will be achieved by heating at an elevated temperature, generally  $\geq 95^{\circ}\text{C}$  and hybridization proceeding at about  $60^{\circ}\text{C}$ , where various buffers may be employed, as well as salt concentrations, to provide the necessary stringency. Usually, 5 fairly high stringencies will be employed, generally at least about equivalent to or greater than about 0.1 M NaCl, usually about 1 M NaCl.

After melting and reannealing, there will be a substantial enrichment of target DNA in the total double-stranded DNA, 10 since the target DNA will not be inhibited from self-annealing due to the lack or relative deficiency of complementary sequences present in the driver DNA.

Overhangs are then filled in by employing any convenient DNA polymerase, e.g., Taq DNA polymerase, in the presence of the 15 four nucleotides, whereby only double-stranded, self-reannealed tester DNA will have filled-in adaptors at each end of the amplicon. Since the driver DNA does not inhibit target DNA from self-annealing, while the driver DNA inhibits non-target tester DNA from self-annealing, there is a substantial 20 enrichment in the target DNA as compared to the total tester DNA.

The double-stranded self-reannealed tester amplicon will then be amplified under conventional polymerase chain reaction conditions, usually involving at least about 5 cycles, 25 frequently as many as 10 cycles and usually not more than about 40 cycles, preferably not more than about 30 cycles. The amplification may be interrupted about midway and single-stranded DNA degraded using an appropriate nuclease. Various nucleases may be employed, particularly mung bean nuclease.

30 The resulting double-stranded DNA mixture may then be digested with a restriction endonuclease which removes the adaptors from the tester DNA. The tester DNA may be separated from the adaptor sequence, using any convenient means which permits separation by size. Gel filtration or gel 35 electrophoresis may be conveniently employed. The amplicons may then be ligated to a second set of adaptors, usually different from the first or previous set and the cycle of melting in the presence of excess driver amplicon, annealing,

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filling in overhangs, and PCR amplification repeated. Later cycles may rely on the previous adaptors. In the subject process, this cycle may be repeated one or more times, there usually being at least 2 rounds or repetitions and not more  
5 than about 6 rounds, usually 2 to 4 rounds being sufficient.

It will frequently be of interest to carry out the process more than once, where different restriction endonucleases are employed for each study. In this way, different amplicons will be obtained and one may obtain different information.  
10 Depending upon the purpose for the process, two or more restriction endonucleases may be utilized in separate preparations of the amplicons. One may also compare the probes obtained with different restriction endonucleases to determine if they overlap, bind to genomic DNA sequences which are  
15 proximal, are part of the same gene or polymorphic region, and the like.

In carrying out the process, the first round is mainly subtractive. Subsequent rounds have a greatly-increased component of kinetic enrichment. For example, if target DNA  
20 is equimolar with respect to tester DNA (i.e. a single copy), and if driver amplicon is taken in N-fold excess to tester amplicon, assuming virtually complete reannealing of driver amplicon, target will be enriched N times after the first round. After the second round, target will be enriched  $N^2$   
25 multiplied by a factor due to the subtractive component, and after the third time, at least the square of that. If N is 50, at the end of the second round, target will be enriched by about  $10^4$ , and at the end of the third round, on the order of  $10^8$ . In general a single cycle of subtraction can be expected  
30 to yield enrichments of target in the order of  $fN$ , where N is the molar excess of driver amplicon to tester amplicon and f is the fraction of driver amplicon that reanneals.

The resulting target DNA or difference product may be further enriched for probes defining differences between the DNA  
35 sources. Conveniently, the sequences may be cloned and then screened using Southern blots or other technique for determining complementation against tester and driver

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amplicons. Those clones which hybridize to tester amplicons and not driver amplicons may then be used further.

The resulting target DNA may be used as probes to identify sites on the tester DNA genome which differ from the driver  
5 DNA. For this purpose, they may be labeled in a variety of ways, such as with radioactive labels, biotin, fluorescers, etc. Desirably, in order to obtain substantially homogeneous compositions of each of the target amplicons, the target amplicons may be cloned by inserting into an appropriate  
10 cloning vector for cloning in a prokaryotic host. If desired, the cloned DNA may be sequenced to determine the nature of the target DNA. Alternatively, the cloned DNA may be labeled as described above, and used as probes to identify fragments in libraries carrying the target DNA. The target DNA may be used  
15 to identify the differences which may be present between the two sources of DNA.

Where a plurality of probes for target DNA are obtained, they may be referred to as putative probes until established as true probes. Conveniently, the sequences may be cloned and then  
20 screened using Southern blots or other technique for determining complementation against tester and driver amplicons. Thus, the group of probes may include hybridizing sequences which hybridize to both driver and tester DNA. One can quickly determine those putative probes which do not  
25 distinguish between driver and tester DNA by hybridizing, e.g. Southern hybridizing, the probe to driver and tester amplicons. Where the putative probe binds to both driver and tester amplicons, the probe may be discarded. Those clones which hybridize to tester amplicons and not driver amplicons  
30 may then be used further. This screen is particularly useful where at least 5, more usually at least 10 putative probes are obtained.

In pedigree analysis, the subject process may be used to define sequences which are present in one member of a family  
35 and not present in another. In this way, one may then compare other members of the family as to whether they carry the same DNA or it is absent. This may find use in forensic medicine, where there may be an interest in the relationship between two

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individuals, a sample obtained from a source and an individual, or the like.

The subject method can also be used to construct libraries of probes for genetic polymorphisms, which may be referred to as PARFs, which is operationally defined as a polymorphic restriction endonuclease fragment, present in the amplified DNA from one genome and not present in the amplified DNA from a different genome from a like organism. For example, if one of two BamHI sites flanking a short BamHI fragment in tester DNA is absent in both alleles from driver DNA, leading to only large BamHI fragments in driver, the short BamHI fragment of tester will be present in its BamHI amplicon, but absent in the BamHI amplicon of the driver. Thus, the restriction fragment would directly lead to a probe which will distinguish between the two genomes.

It should be appreciated, that where the amplicons are cloned, there may be substantial redundancy in individually-picked clones. Therefore, the efficiency of selecting different probes will vary substantially depending upon the frequency in which the amplicon was present in the mixture prior to cloning, which may be as a result of the varied efficiency of amplification, or other artefacts which are built into the methodology.

The subject method can be used to isolate probes for pathogens, where DNA which is suspected of being infected may be compared to DNA which is believed to be uninfected. For example, if one were interested in a virus which is tropic for a particular cell type or tissue, e.g., HIV for T-cells and macrophages or hepatitis B virus for liver, one could take tissue from the source suspected of infection for which the virus is tropic and tissue from another site in the same individual, where such virus should not be present. By carrying out the process, one should obtain probes which would be specific for the virus, since by appropriate selection of the sources of the cells, one would not anticipate any other differences.

A limitation of the subject process, which will be applicable to viruses, as well as other situations, is that the population

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carrying the target DNA should be a reasonable proportion of the total number of cells from which the tester DNA is derived. As indicated above, where one is interested in the presence of integrated pathogenic DNA, it may be that only a small  
5 proportion of these cells in the tissue are infected. It may, therefore, be desirable to normalize the tester sequences, in order to equalize the concentrations of all tester sequences, prior to the subtractive and kinetic enrichment (Patanjali et al., *Proc. Natl. Acad. Sci. USA* 88, 1943 [1991]).

10 Application of RDA to the discovery of pathogens desirably requires a careful matching of the polymorphisms from the infected and uninfected DNA sources. Tester and driver DNA can derive from the same individual, if the individual is not a genetic mosaic. These DNAs should not derive from unrelated  
15 individuals, as the abundant polymorphic differences in their DNAs would obscure the detection of the pathogen. However, the uninfected DNA source (driver) could, in principle, come from an identical twin, or be the pooled DNA from the parents of the infected individual, because virtually all of the DNA  
20 restriction fragments found in the genomic DNA of the infected individual can be expected to be present in at least one parent DNA.

The subject methodology may also be applied to detecting genomic alterations occurring in cancer cells. These could be  
25 of three distinct types: those that result in loss of restriction endonuclease fragments, such as might occur from deletions or gene conversions extending over heterozygous polymorphisms; those that produce new restriction endonuclease fragments, such as might result from point mutations or genomic  
30 rearrangements; and those that result in the amplification of DNA, usually incorporating a gene. In the second and third cases, RDA could be applied without modifications using DNA from cancer cells as tester and normal DNA as driver. However, the presence of normal stroma in a cancer biopsy could  
35 interfere with the detection of loss of genetic information in the cancer cell. Hence, either cultures of cancer cells or highly-purified cancer cells obtained by physical separation would be needed as the source for tester in the first case.

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These restraints do not apply to the detection of genomic rearrangements. Genomic rearrangements, including translocations, insertions, inversions and deletions, will result in the creation of new restriction endonuclease fragments bridging the site of the rearrangement. Some of these bridging fragments may be amplifiable, while at least one of the fragments from which they derive in normal DNA is not. Such bridging fragments would be discoverable by RDA, when DNA from the tumor is used for preparation of tester amplicons and DNA from normal tissue of the same individual is used for preparation of driver amplicons.

The different-sized restriction endonuclease fragments created by genomic rearrangements may be exploited another way. Fractionated size classes from tumor DNA digests will sometimes contain sequences that are not present in comparable-size classes from normal DNA. Using the former as tester and the latter as driver, one can prepare amplicons after cleavage with a second restriction endonuclease and compare these by RDA in order to clone amplifiable restriction endonuclease fragments in proximity to the point of genetic rearrangement. With either of the above-indicated methods, the presence of normal cells among the tumor cells will not obscure the detection of probes for the rearrangement.

In the final situation, DNA amplification, it appears that the detection of amplification is a result of kinetic enrichment during RDA. Being able to detect amplified sequences can find application in cancer prognosis, since it has been found that amplification of oncogenes indicates a poor prognosis.

When RDA is applied to different individuals, it will yield a collection of polymorphisms of a type, which has been previously referred to as PARFs. Thus, RDA can be used for generating new sets of polymorphisms, not only for species that have not previously undergone extensive molecular genetic characterization, but also for well-studied species as humans and mice. Since PARFs most often detect binary polymorphisms, they can serve as a panel of probes that can be used with a standardized format for genetic typing.

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In yet another application, RDA can yield probes for PARFs present in the DNA of an individual from a founder group affected by some autosomal dominant inherited disorder (the tester), but absent in the DNA of an individual from a normal group (the driver). Conversely, RDA can yield probes for PARFs present in the DNA of a normal individual (the tester), but absent in the DNA of an individual from the founder group affected by a recessive inherited disorder (the driver). Combined with methodologies for coincidence cloning (Brooks and Porteous, *Nuc. Acid Res.* 19, 2609 [1991]), such applications can accelerate the discovery of probes for rare PARFs in linkage disequilibrium with the dominant locus, or the absence of common PARFs in linkage disequilibrium with the recessive locus.

In many laboratory animals and plants there are congenic strains, where a particular gene has been transferred from one genetic background onto another by successive generations of backcrossing. Such strains will be genetically identical except in a relatively small region surrounding the gene of interest. The region will be typically small enough to permit chromosomal walking to the target gene, but large enough for the needs of the subject methodology.

The subject methodology may be applied to the discovery of polymorphisms that are genetically linked to an inherited trait such as a disease susceptibility or a behavioral abnormality. To utilize the subject methodology for this purpose, it is desirable to use pools of DNAs from a group of individual for use as either tester, driver or both. When used this way, the method may yield probes that detect polymorphic alleles that are present in one group and not in another. In particular, when such pools are used as driver, the probes obtained for restriction endonuclease polymorphisms ("PARFs") that distinguish tester from all individuals in the driver pool. When pools are used as tester, the method yields PARFs that distinguish at least one member of the tester pool from the driver individual. In the most challenging example, when both tester and driver are pooled DNAs from groups of individuals,



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the method yields PARFs that distinguish at least one member of the tester group from all members of the driver group.

Pooling may be demonstrated in a variety of situations. One application uses transmission genetics to produce a collection  
5 of siblings with the property that their pooled DNA is homozygous in the region of a target gene but heterozygous elsewhere in the genome. As an illustration, if two inbred strains differ at a target locus L of interest, one strain A carries a recessive allele ( $a^-$ ) and the other strain B carries  
10 a dominant allele ( $a^+$ ), for tester one can use strain B, while for Driver, one performs an F2 intercross between the strains, selects k progeny showing the recessive phenotype, and mixes their DNA together. When employing the subject method, B alleles should be subtracted everywhere in the genome except  
15 in a region around L.

The targetting of the method can be further improved where the locus L has been genetically mapped between two flanking genetic markers, X and Y. For the driver, one can select 1/2  
20 k progeny in which a crossover had occurred between X and L and 1/2 k progeny in which a crossover had occurred between L and Y. this would guarantee that the proportion of B alleles is 25 % at X and Y. This ensures that the region over which the proportion of B alleles is very low is restricted to the interval X -Y.

25 The pools may be of various sizes depending on the source of DNA. From large genomes, such as mammalian and plant genomes, generally a pool as small as 8 different sources may be employed, usually 10, and generally not more than 50, usually not more than about 20.

30 Other applications may involve spontaneous germ line genomic rearrangements. The genome of such an infected individual will include restriction endonuclease fragments that are present in neither parent. This situation is analogous to genetic rearrangements occurring in cancer cells, which has been  
35 previously discussed.

To ensure that the subject process has operated properly, it will normally be desirable to test candidate difference products (target DNA) for its presence or absence in tester and

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driver amplicons. Also of concern will be the presence of flora, which may contaminate tester, but is not present in driver. Genetic mosaicism will also interfere with the subject methodology. However, in a wide variety of contexts, the  
 5 subject method will efficiently provide sequences which can be used for analyzing differences between two genomes as a result of a wide variety of events.

The following examples are offered by way of illustration and not by way of limitation.

10

EXPERIMENTAL

Preparation of Amplicons. 10 µg of high molecular weight DNA purified from the lymphoid cell line DRL 484 (a gift of T. Caskey, Baylor College) was used for preparation of driver amplicons and 10 µg of the same DNA, containing equimolar  
 15 amounts of target (120 pg of adenovirus-2 DNA and/or 160 pg of λ phage DNA, both from New England Biolabs) was taken for preparation of tester amplicons. Both tester and driver DNA samples were digested with restriction endonuclease (New England Biolabs) and 1 µg of each DNA digest was mixed with  
 20 0.5 nmoles of 24-mer and of 12-mer unphosphorylated oligonucleotides (set 1, see Table 1) in 30 µL of T4 DNA ligase buffer (New England Biolabs).

25 **Table 1.** Sequences of Primers Used for Representational Difference Analysis.

Primer Set	Name	Sequence
1	R Bgl 24	5'-AGCACTCTCCAGCCTCTCACCGCA-3'
	R Bgl12	5'-GATCTGCGGTGA-3'
2	J Bgl24	5'-ACCGACGTCGACTATCCATGAACA-3'
	J Bgl12	5'-GATCTGTTTCATG-3'
3	N Bgl24	5'-AGGCAACTGTGCTATCCGAGGGAA-3'
	N Bgl12	5'-GATCTTCCCTCG-3'
1	R Bam24	5'-AGCACTCTCCAGCCTCTCACCGAG-3'
	R Bam12	5'-GATCCTCGGTGA-3'

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Primer Set	Name	Sequence
2	J Bam24	5'-ACCGACGTCGACTATCCATGAACG-3'
	J Bam12	5'-GATCCGTTCATG-3'
3	N Bam24	5'-AGGCAACTGTGCTATCCGAGGGAG-3'
	N Bam12	5'-GATCCTCCCTCG-3'
1	R Hind24	Same as R Bgl24 (see above)
	R Hind12	5'-AGCTTGCGGTGA-3'
2	J Hind24	Same as J Bgl24 (see above)
	J Hind12	5'-AGCTTGTTTCATG-3'
3	N Hind24	5'-AGGCAGCTGTGGTATCGAGGGAGA-3'
	N Hind12	5'-AGCTTCTCCCTC-3'
1	Seq24	5'-CGACGTTGTAAAACGACGGCCAGT-3
	Rev25	5'-CACACAGGAAACAGCTATGACCATG-3'

Primer set 1 (R series) is used for representations, and sets 2 (J series) and 3 (N series) are used for odd and even hybridization/amplifications, respectively. Oligonucleotide design was checked for the absence of strong secondary structure using the OLIGO computer program (National Biosciences).

Oligonucleotides were annealed by cooling the mixture gradually from 50°C to 10°C for one hour and then ligated to human DNA fragments by overnight incubation with 400 U of T4 DNA ligase at 16°C. Following ligation, both tester and driver DNA samples were amplified. Each of 10 tubes taken for preparation of driver amplicons and 2 tubes used for preparation of tester amplicons contained in a volume of 400 µl: 67 mM Tris-HCl, pH 8.8 at 25°C, 4 mM MgCl<sub>2</sub>, 16 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM β-mercaptoethanol, 100 µg/ml bovine serum albumin, 200 µM (each) dATP, dGTP, dCTP, and dTTP, 1 µM 24-mer primer and 80 ng of DNA with ligated adaptors. The tubes were incubated for 3 min. at 72°C in a thermal cycler (Perkin Elmer Cetus), 15 U of Taq polymerase (AmpliTaq, Perkin Elmer Cetus) was added, the reactions were overlaid with mineral oil, incubated for 5 min. to fill in 5' protruding ends of ligated adaptors, and amplified for 20 cycles (each cycle including 1 min. incubation at 95°C and 3 min. at 72°C, with the last

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cycle followed by an extension at 72°C for 10 min.). After amplification both driver and tester amplicons were digested with the same restriction endonuclease (10 U/μg) to cleave away adaptors. 10 μg of tester amplicon DNA digest was electrophoresed through 2% NuSieve agarose (low melting point, FMC Bio Products), and DNA fragments (150-1500 bp) were recovered after melting of the agarose slice and Qiagen-tip20 chromatography (Quiagen Inc.) to remove adaptors. These fragments were ligated to a new set of adaptors (primer set 2, see Table 1) in preparation for the first round of hybridization and amplification.

DNA Hybridization and Amplification Step. 0.5 μg of the tester amplicon ligated to adaptors and 40 μg of driver amplicon DNA were mixed, ethanol precipitated, dissolved in 4 μl of 3xEE buffer (Straus and Ausbel, *Proc. Natl. Acad. Sci. USA* 87, 1889 [1990]) and overlaid with 30 μl of mineral oil (Perkin Elmer Cetus). Following heat denaturation 1 μl of 5 M NaCl solution was added and DNA was hybridized for 20 h at 67°C. At the end of hybridization, 1/10th part of the resulting DNA was incubated with 15 U of Taq polymerase (5 min., 72°C) in 400 μl of PCR mixture without primer to fill in ends of reannealed tester, and then amplified for 10 cycles (1 min. at 95°C, 3 min. at 70°C, followed by 10 min. extension for the last round) after addition of the same 24-mer oligonucleotide to which tester was ligated. Single stranded DNA molecules present after amplification were degraded by 30 min. incubation with 20 U of mung bean nuclease (New England Biolabs) in a volume of 40 μl as recommended by the supplier followed by 5-fold dilution of the sample in 50 mM Tris-HCl, pH 8.9 and heat inactivation of enzyme (95°C, 5 min.). 40 μl of the solution was amplified for 15-20 cycles under the same conditions as before the mung bean nuclease treatment. Amplified DNA (3-5 μg) was digested with the original restriction endonuclease and 200 ng of the digest was ligated to the third adaptor set (see Table 1). 50-100 ng of this DNA was mixed with 40 μg of driver amplicon and the hybridization and amplification procedures were repeated as in the first

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cycle. 200 ng of the digest obtained after the second hybridization/amplification step was then ligated to the second set of adaptors and 100-400 pg of this material together with 40 µg of driver amplicon was taken for the third round of  
5 hybridization, with the final amplification after mung bean nuclease digestion for 20-25 cycles. A fourth hybridization/amplification step was performed after taking 5 pg of material from the third round ligated to adaptors of the third set and mixing it with 40 µg of driver amplicon.

10 Example 1. Representational Difference Analysis with Viral DNAs Added as Targets.

Single-copy levels of adenovirus and/or bacteriophage λ DNA was added to human DNA to create a model tester, and used with the same human DNA without viral DNA as driver. BglII  
15 amplicons from human DNA with adenovirus and λ DNAs as targets or HindIII amplicons with λ DNA as target were prepared. With BglII amplicons, small λ and adenovirus fragments were the major difference products, even after two rounds, as evidenced by agarose gel electrophoresis. This represented an enrichment  
20 of  $> 5 \times 10^6$ -fold from the starting material and a probable enrichment of about  $4 \times 10^5$ -fold from amplicons.

The enrichment from HindIII amplicons was not as effective. The λ HindIII fragment was greatly enriched after the third round as evidenced by blot hybridization, but still not to  
25 homogeneity. After the fourth round the expected target fragment was purified to near homogeneity. The difference between the experience with the HindIII restriction endonuclease and the BglII restriction endonuclease may be related to the greater sequence complexity of the HindIII  
30 amplicons. When the complexity of the driver is too high, subtractive and kinetic enrichments are diminished and competing processes may dominate. The competing processes may involve the emergence of efficiently-amplified repetitive sequences in tester.

35 Example 2. Representational Difference Analysis of DNAs from Two Individuals.

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Driver and tester amplicons were prepared from human lymphoblastoid cell cultures GM05901 and GM05987, respectively (Amish Pedigree 884, Human Genetic Mutant Cell Repository, Camden, NJ). Amplicons were prepared after cleavage with

5 BamHI, BglII or HindIII. Difference products between amplicons were obtained as described above and size fractionated by gel electrophoresis. A discrete but complex pattern of bands was observed in each case. After three

10 hybridizations/amplifications, difference products were cloned into plasmids. For each difference product, three probes were picked for blot hybridization analysis. It was found that all of them were polymorphic within the Amish family data. BamHI difference products were analyzed in greatest detail.

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Table 2. Screening for Presence of BamHI PARFs in 17 Human DNA Samples.

Probe Number (%)	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	Length of alleles in kbp	
																		Large	Small
1 (15.5)	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15	0.61, 0.67 <sup>(a)</sup>
11 (14.4)	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	15	0.6
6 (8.9)	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	3.5	0.58
19 (5.5)	-	+	+	-	+	+	+	+	+	+	-	+	-	-	+	+	+	15	0.51
17 (4.4)	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	8	0.48
22 (4.4)	-	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+	6.5	0.67
8 (3.3)	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	+	ND	0.62
24 (3.3)	-	+	-	+	+	+	+	+	-	+	+	-	+	+	-	+	-	>50	0.65
26 (3.3)	-	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	-	6, 5 <sup>(b)</sup>	0.65
9 (2.2)	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	ND	0.47
65 (2.2)	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	4	0.74
3 (1.1)	-	+	+	+	-	+	+	+	+	+	+	-	+	-	+	+	+	ND	0.5

5

10

15

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BamHI amplicons were prepared from DNA from seven Amish pedigree lymphoblastoid cell cultures, GM05901 (driver), GM05987 (tester), GM05918, GM05961, GM05963, GM05993, GM05995 (columns A-G), five different placentas (columns H-L), three lymphoblastoid cell lines established from the biopsies of leukemic patients (columns M, N, O) and two fibroblast cell cultures, DRL 484, and DRL 569 (a gift of T. Caskey, Baylor College) established from the biopsies of DMD patients (columns P, Q), transferred to GeneScreen membrane, and hybridized to the indicated probes. "%" indicates the percent of clones in a BamHI PARF collection of difference products cloned after three hybridization-amplification steps that hybridized to the indicated clone. "+" means that the small BamHI PARF allele was present in the sample (i.e. the probe hybridized to a band of the correct size in the amplicon); "-" means that the small allele was not detected. See Fig. 3C for a sample of the actual data. The lengths of the alleles hybridizing to PARFs are indicated, where known. "ND" means not determined.

(a) Two different small alleles were found in the human population.

(b) Two different large alleles were found in the human population.

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Of 20 randomly-picked clones, 12 unique clones remained after removing redundancies, and the inserts from 9 of these were used as probes in Southern blots of tester, driver and 5 other members of the family (GM05918, GM05987 [tester], GM05901 [driver], GM05961, GM05963, GM05993, and GM05995 from Amish pedigree 884). All probes detected small BamHI fragments in the tester (Table 2, col. B) and only large BamHI fragments in the driver (Table 2, col. A). The blot hybridization pattern for each probe was completely consistent with a Mendelian pattern of inheritance. The results demonstrate that collections of probes for restriction endonuclease fragment polymorphisms may be obtained between two related individuals.



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Each of the BamHI probes derived from the above experiment was also used in blot hybridizations to amplicons from the family and 10 other unrelated human DNAs extracted from cell lines or placentas (Table 2). Complete concordance between this method and Southern blotting of total genomic DNA was found. These results support the conclusion that the probes which detect polymorphisms within the Amish family will also detect polymorphisms in the human population at large. As indicated previously, these polymorphisms are referred to as PARFs (polymorphic amplifiable restriction endonuclease fragments).

The probes for PARFs are not equally abundant in the difference product. To obtain a measure of this unevenness, each cloned BamHI PARF was hybridized to a grid of 90 individually randomly-picked clones from the difference product of the two siblings, and its frequency in the collection was determined (see percent value in Table 2). From a total of 90 randomly-picked elements, only 20 distinct polymorphic probes were present.

It should be noted that the protocol was designed for the detection of a small number of differences between two nearly-identical genomes. Where probes for polymorphic loci are deliberately sought, more representative difference products can be generated by diminishing the number of rounds of hybridization/amplification, increasing the complexity of the representation and/or decreasing the total number of PCR cycles.

\*\*\* The following is an exemplary protocol used in the following examples, except where otherwise indicated.

#### DIFFERENCE ANALYSIS PROTOCOL

##### I. Preparation of amplicons

##### 1. Restriction of DNA.

a. Digest 10  $\mu$ g of Driver and Tester DNA with a restriction enzyme chosen for representation, taking 10 U/ $\mu$ g of high molecular weight DNA.

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- b. Extract with equal volumes of phenol and phenol/chloroform.
- c. Add NaOAc to final concentration 0.3 M, EtOH ppt., wash with 70% EtOH, dry in vacuo and resuspend at 0.1 mg/ml.
- 5 2. Purification of oligonucleotides
- a. Attach Sep-Paq cartridge (Waters, Millipore) to 5 ml syringe and wash it with 10 ml of acetonitrile and 10 ml of water.
- b. Load 20 OD<sub>260</sub> of the oligonucleotide in 2 ml of  
10 water, wash with 10 ml of water and elute with 60% MeOH, collecting 7 fractions in Eppendorf tubes (3 drops per each tube).
- c. Measure DNA concentration of 200 fold dilutions at  $\lambda=260$  nm, combine DNA containing fractions (approx. 500  $\mu$ l) and  
15 concentrate by liophylization up to 200-300  $\mu$ l.
- d. EtOH ppt. (use 4 vol. of EtOH) after addition of 1/10 vol. 3 M NaOAc, wash with 100% EtOH, dry, resuspend at 62 pmol/ $\mu$ l (12 OD<sub>260</sub>/ml for 24-mers and 6 OD<sub>260</sub>/ml for 12-mers).
3. Ligation of adaptors
- 20 a. Mix: 20  $\mu$ l (2  $\mu$ g) of Driver or Tester DNA digest, 15  $\mu$ l of each 12-mer and 24-mer (primer set 1),
- 4  $\mu$ l of ddH<sub>2</sub>O,  
6  $\mu$ l of 10 x Ligase buffer.
- 25 b. To anneal the oligonucleotides, place the tubes in a heating block (Termoline DriBath, holes filled with glycerol) at 50-55°C and then place the block in a cold room for approx. 1 h, until the temperature will decrease to 10-15°C.
- c. Place the tubes on ice for 3 min., add 2  $\mu$ l  
30 (400 U/ $\mu$ l) of T4 DNA ligase, and incubate overnight at 12-16°C.
4. PCR
- a. Add 940  $\mu$ l of TE (10 mM Tris-HCl, pH 8.0/1 mM EDTA) plus tRNA (20  $\mu$ g/ml) buffer to each ligate to make a dilution.
- b. Makes 2 tubes of PCR mix for preparation of Tester  
35 amplicon and 10 tubes for preparation of Driver amplicon, each containing:
- 80  $\mu$ l of 5 x PCR buffer (335 mM Tris-HCl, pH 8.8 at 25°C, 20 mM MgCl<sub>2</sub>,

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80 mM  $(\text{NH}_4)_2\text{SO}_4$ , 50 mM  $\beta$ -mercaptoethanol, 0.5 mg/ml of bovine serum albumin)

32  $\mu\text{l}$  of chase solution (4 mM of each dATP, dGTP, dCTP, dTTP)

- 5           8  $\mu\text{l}$  of 24-mer oligonucleotide (primer set 1)  
          240  $\mu\text{l}$  of  $\text{ddH}_2\text{O}$ .

c. Add 40  $\mu\text{l}$  of DNA ligate dilution (80 ng) in each tube and place the tubes in a Thermocycler (Perkin Elmer Cetus) at 72°C.

- 10          d. To fill-in 5'-protruding ends of the ligated adaptors, add 3  $\mu\text{l}$  (15 U) of AmpliTaq DNA polymerase in each tube (use Aerosol Barrier Pipet Tips), mix, overlay with 110  $\mu\text{l}$  of mineral oil and incubate for 5 min.

- e. Amplify for 20 cycles (1 min. at 95°C and 3 min.  
15 at 72°C) with the last cycle followed by extension at 72°C for 10 min.

5. Restriction of amplicons

- a. Remove mineral oil, combine the contents of each of 2 PCR tubes in Eppendorf, extract with 600  $\mu\text{l}$  of phenol and  
20 phenol/chloroform.

- b. Add 1/10 vol. of 3 M NaOAc and equal volume of isopropanol, incubate for 15 min. in ice bath, spin, wash, dry. Resuspend Driver and Tester amplicons in TE at concentration 0.2-0.4 mg/ml (expecting 10-20  $\mu\text{g}$  of DNA amplicon from one PCR  
25 tube), check DNA concentration using EtdBr solution (2  $\mu\text{g}/\text{ml}$ ).

c. Digest both Driver DNA (200  $\mu\text{g}$ ) and Tester DNA (20  $\mu\text{g}$ ) with initially chosen restriction endonuclease in order to cleave the adaptors, extract and iProOH ppt. as above.

- d. Resuspend Driver amplicon DNA digest in TE at  
30 approx. 1 mg/ml and Tester amplicon DNA digest at 0.2-0.4 mg/ml. Measure Driver and Tester DNA concentrations by EtdBr fluorescence and agarose gel electrophoresis. Adjust Driver DNA concentration to 0.5 mg/ml and Tester DNA concentration to 0.1 mg/ml.

- 35          6. Change of adaptors on Tester amplicon

a. Load 10  $\mu\text{g}$  of Tester amplicon DNA digest on 2% NuSieve agarose gel (low melting point, FMC Bioproducts).

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b. Cut agarose slice (0.2-0.4 g) containing fragments 150-1500 bp in length and put it in a 5 ml Falcon tube. Add 0.4 ml of 0.5 M MOPS pH 7.0, 0.4 ml 5 M NaCl and 3 ml of ddH<sub>2</sub>O.

c. Mix, melt at 72°C in a heating block for 10 min.,  
5 repeat this step one more time.

d. Pass warm solution (30-50°C) through Qiagen-tip20 (Qiagen Inc.), elute and precipitate DNA material as recommended by the supplier. Dissolve DNA pellet in 30 µl of TE buffer, check DNA concentration by EtdBr fluorescence,  
10 adjust to 0.1 mg/ml.

e. Ligate 2 µg of purified Tester DNA amplicon DNA digest to primer set 2, as described above, dilute with TE plus tRNA up to 10 µg/ml (25 µg/ml for Hind III representation).

## II. DNA hybridization/amplification steps

### 15 1. Hybridization 1.

a. Mix 80 µl of Driver amplicon DNA digest (0.5 mg/ml) and 40 µl of diluted Tester amplicon ligate (0.4 µg for representations made with most six cutters, 1 µg for Hind III representation), extract once with phenol/chloroform.

20 b. Add 30 µl of 10 M NH<sub>4</sub>OAc and 380 µl (2.5 vol.) of EtOH, chill at -70°C for 10 min. incubate at 37°C for 2 min., spin, wash twice with 70% EtOH, dry.

c. Resuspend the pellet in 4 µl of EE x 3 buffer (30 mM EPPS from Sigma, pH 8.0 at 20°C, 3 mM EDTA) by vortexing  
25 for 2 min., spin the sample to the bottom and overlay with 35 µl of mineral oil.

d. Denature DNA for 3-4 min. at 98°C in a heating block, carefully add 1 µl of 5 M NaCl to the DNA drop and incubate at 67°C for 20 h.

### 30 2. Selective amplification

a. Remove oil, add 8 µl of tRNA solution (5 mg/ml), mix, add 390 µl of TE buffer and mix again.

b. To fill-in the adapter ends, make 2 tubes with 360 µl of PCR mix (see above), not including 24-mer primer.

35 Add 40 µl of hybridized DNA dilution in each tube, place in Thermocycler at 72°C, add 3 µl of AmpliTaq DNA polymerase, mix, and incubate for 5 min. Add 10 µl of 24-mer primer (set 2),

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mix, overlay with mineral oil and perform 10 cycles of PCR as above. For J Bgl 24 primer lower annealing temperature (70°C) is required.

c. Phenol and phenol/chloroform extract, iProOH ppt.  
5 as above, dissolve the pellet in each tube in 20  $\mu$ l of ddH<sub>2</sub>O, combine.

d. Take 20  $\mu$ l of the amplified difference product 1, add 20  $\mu$ l of 2 x mung bean nuclease buffer and 2  $\mu$ l of mung bean nuclease (10 U/ $\mu$ l, NEB), incubate at 30°C for 30 min. Add  
10 160  $\mu$ l of 50 mM Tris-HCl pH 8.9, inactivate the enzyme by 5 min. incubation at 98°C. Prepare 2 tubes with a PCR mix (360  $\mu$ l), containing J 24-mer primer, add 40  $\mu$ l of MBN-treated difference product in each tube and make PCR for 15 cycles as above.

15 e. Run 10  $\mu$ l of the amplificate on a 2% agarose gel, estimate the quantity of DNA (usually 0.1-0.3  $\mu$ g) and, if necessary to improve the yield, make 2-4 additional cycles after addition of 3  $\mu$ l of fresh AmpliTaq DNA polymerase.

3. Change of adapter on a difference product

20 a. Extract with phenol and phenol/chloroform, iProOH ppt. as above and dissolve the pellet at approx. 0.1 mg/ml. Determine DNA concentration by EtBr fluorescence, adjust up to 0.1 mg/ml.

b. Digest difference product with chosen restriction  
25 enzyme (10 U/ $\mu$ g), extract as above and EtOH ppt., wash, dry, dissolve at 20 ng/ $\mu$ l.

c. Take 10  $\mu$ l (200 ng) of DNA solution and directly ligate to adapter 3 (primer set 3) in a volume 60  $\mu$ l as described above. Dilute the ligated difference product up to  
30 1.25 ng/ $\mu$ l (2.5 ng/ $\mu$ l for Hind III representation) with 100  $\mu$ l of TE buffer containing tRNA (20  $\mu$ l for Hind III).

4. Subsequent hybridization/amplification steps

a. For second hybridization mix 40  $\mu$ l (50 ng) of adapter ligated difference product (100 ng for Hind III  
35 representation) and 80  $\mu$ l (40  $\mu$ g) of Driver amplicon DNA digest. Proceed through hybridization/amplification step as above.

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b. For third hybridization/amplification step take 100 pg of difference product 2 ligated to the adapter 2 (400 pg for Hind III representation), making final amplification after MBN treatment for 20 cycles (25 for Hind III representation).

- 5 c. For Hind III representation sometimes the fourth hybridization/amplification step is needed. Take 5 pg of difference product 3 ligated to adapter 3 with final amplification for 27 cycles.

### III. Cloning and analysis of difference products

10 1. Cloning

a. Take 10  $\mu$ g of the difference product after the last hybridization/amplification step, digest with chosen restriction enzyme, extract with phenol and phenol/chloroform, EtOH ppt.

- 15 b. Dissolve obtained DNA in 100  $\mu$ l of TAE buffer and make 2% low melting point (LMP) gel electrophoresis and DNA purification as above.

c. Dissolve digested difference product in 30  $\mu$ l of TE buffer, check the concentration and dilute an aliquot (2-5  $\mu$ g) up to 10 ng/ml with tRNA containing TE buffer.

- 20 d. To ligate the difference product in a plasmid vector mix:

1  $\mu$ l of 10 x ligase buffer,

6  $\mu$ l of ddH<sub>2</sub>O,

- 25 1  $\mu$ l (10 ng) of gel-purified difference product DNA digest,

1  $\mu$ l (40 ng) of any pUC-derived vector, digested with chosen restriction enzyme and dephosphorylated,

1  $\mu$ l (400 U) of T4 DNA ligase.

- 30 Incubate for 1-3 h at 16°C and dilute by addition of 70  $\mu$ l of tRNA containing TE.

e. Transform the competent DH 5 $\alpha$  cells in a standard way. Plate on LB agar containing ampicillin, X-Gal, and IPTG.

#### 2. PCR amplification of cloned inserts

- 35 a. Prepare PCR tubes each containing 100  $\mu$ l of standard PCR mixture and sequencing and reverse sequencing

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primers (seq. 24 and rev. 25, respectively, see Table) (500 pmol of each per tube).

b. Pick and transfer one white bacterial colony in each tube, vortex and place in Thermocycler at 95°C for 5 min.

5 c. Lower the temperature by switching to 72°C, add 1 µl (5 U) of AmpliTaq polymerase, mix, overlay with mineral oil and perform PCR for 30 cycles (1 min. at 95°C, 3 min. at 72°C) with final extension at 72°C for 10 min.

d. Analyze the yield and the size of the amplified  
10 fragments by 2% gel electrophoresis of 5 µl aliquots. Purify chosen DNA fragments by Qiagen-tip20 chromatography, iProOH ppt., wash, dry and dissolve in 30 µl of TE.

e. Determine DNA concentration by EtdBr fluorescence. For blot hybridizations dilute 1-2 µg of each fragment up to  
15 10 µg/ml with tRNA containing TE buffer.

Example 3. Application of RDA to isolating DNA probes that detect gene amplification in cancers. When tumor DNA was taken as tester and normal DNA from humans was taken as driver, RDA yielded difference products that hybridized to  
20 amplified sequences in the tumor DNA. This is an unanticipated result, the probable consequence of the kinetic enrichment during RDA. Probes that detect amplified sequences in human cancers are of clinical value, since the presence of such sequences usually indicates a poor prognosis. For example,  
25 amplification of N-myc or the NEU oncogenes indicates poor prognosis for neuroblastoma or breast cancer, respectively.

Difference products were found when DNA from a melanoma cell line or DNA from a small cell lung cancer cell line was used as tester and normal DNA from the individual donors,  
30 respectively, was used as driver. The difference products for the 1st, 2nd and 3rd round subtractions of the melanoma were subject to electrophoretic separation, and are shown in Figure 1, right hand panel, lanes a, c and e. The difference products for the 1st, 2nd and 3rd rounds of subtractions of the lung  
35 cancer are shown in lanes b, d and f. Size markers are in lane g, with lengths in basepairs indicated at right. The melanoma cell line was AH-Mel, and the small cell carcinoma cell line

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was H1770. When some of the difference products were used as nucleic acid hybridization probes in genomic blots of restriction endonuclease cleaved human DNA from a variety of cancer cell lines, they detected sequences amplified in the small cell carcinoma cell line (top panel, left side of Figure 1) or the melanoma cell line (middle and lower panel, left side of Figure 1). The probes derived from the RDA analysis of the small cell carcinoma cell line also detect amplified sequences in a neuroblastoma cell line IMR-5 (top panel, left side). The RDA probes were determined to map to human chromosome 2 (small cell lung carcinoma) and chromosome 3 (melanoma) by hybridizing them to a panel of monochromosomal hybrid cells #2 obtained from NIGMS Human Genetic Mutant Cell Repository. No amplifications on chromosome 3 have been previously described.

Next, was determined that driver DNA need not derive from the same individual as the tester. RDA was performed using DNA from the melanoma cell line as tester and using DNA from either the matched individual donor, an unmatched individual, or a pool of 10 unmatched individuals as driver. The same pattern of difference products was found whichever driver DNA was used (see Fig. 2). Thus tester and driver DNAs do not have to derive from the same individual when one is searching for probes that detect amplified DNA present in the tester.

Example 4. The use of RDA to discover new viruses. Human prostate cancer biopsies were analyzed using RDA. DNA extracted from a surgical biopsy of a prostate cancer was used as tester and DNA from normal tissue of the same individual was used as driver. A single difference product was obtained and sequenced. Computer analysis demonstrated that this difference sequence corresponded most closely to a rat LINE element, a member of repeated sequences found interspersed throughout the rat genome (see Fig. 3 for a sequence comparison). Oligonucleotide PCR primers derived from the extreme left hand and right hand sequences of this element were used to demonstrate its presence in various DNAs. Its presence was detected in rat DNA, and two different regions of the human prostate cancer, but not in the DNA from normal tissues of the human in which the cancer arose. Thus genetic information from



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rats has been found in human tissue, presumably through the agency of a virus. The DNA sequences of this presumed virus may be obtained by "chromosomal walking" from the inserted element. One may infer a causal role of this virus in the etiology of this cancer.

Example 5. The use of RDA to isolate probes that detect genetic lesions in cancer. Using DNA from pure or nearly pure (>90%) cancer cells as tester and DNA from normal cells of the respective patient as driver many difference products were obtained. These difference products detected either loss-of-heterozygosity, hemizygous loss on chromosome Y, or homozygous loss in the tumor DNAs. The probes from RDA were mapped to human chromosomes. The results are summarized in Table 3. As tester, DNAs from four different renal cell carcinoma cell lines UOK114, UOK124, UOK132 and UOK112 were used, and one esophageal cancer biopsy, from patient #758. One probe, RCC124.1 (footnote d from Table 3) also detected homozygous loss on chromosome 2 in one additional renal cancer cell line and two bladder cancer cell lines. One probe, RCC132.12 (footnote e from Table 3) also detected homozygous loss on chromosome 9 in two melanomas. One probe, BAR.6 (footnote f from Table 3) also detects homozygous loss on chromosome 3 from several colon cancer cell lines. Probes that detect homozygous loss may be useful to define loci that encode tumor suppressor genes. Methods that detect loss of function of tumor suppressor genes may be useful in the clinical typing of cancers.

Table 3: Application of RDA to the pairs of normal and tumor DNA's (tumor DNA as Driver).

Experiment	RDA fragments		Chromosome s affected <sup>c</sup>
	Selected for initial characterization <sup>a</sup>	Found to be informative <sup>b</sup>	
1. Renal cell carcinoma, cell line UOK114 (male)	12	4 (1/3/0)	3/3,3,10

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	2. Renal cell carcinoma, cell line UOK124 (female)	11	5(2/3/0)	2 <sup>d</sup> /ND
5	3. Renal cell carcinoma, cell line UOK132 (male)	10	9(0/3/6)	-/9 <sup>e</sup> , 9, 5
10	4. Renal cell carcinoma, cell line UOK112 (male)	13	13(0/0/13)	-/-
15	5. Barrett's esophageal cancer, patient #758, sorted nuclei (male)	5	5(1/0/4)	3 <sup>f</sup> /-
	Total	38	23 (4/9/10)	

a. Clones with distinct insert sizes.

20 b. Entries in parentheses (x/y/z) show distribution of fragments according to type of loss, where x is number of probes detecting homozygous loss, y the number detecting loss of heterozygosity, and z the number detecting hemizygous loss from the Y chromosome.

25 c. Chromosomal location of probes, where x/... are the locations of probes detecting homozygous loss, and .../x the locations of probes detecting loss of heterozygosity. ND means not yet determined.

30 d. Probe RCC124.1 also detects homozygous loss in bladder cancer cell lines.

e. One probe, RCC132.12, detected homozygous loss on chromosome 9 in melanomas.

35 f. Probe BAR.6 also detects homozygous loss in four out of seven colon cancer cell lines and one bladder carcinoma cell line.

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40 Example 6. The application of RDA to the analysis of DNA from pools of individual. RDA may be applied to the discovery of polymorphisms that are genetically linked to an inherited trait

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such as a disease susceptibility or a behavioral abnormality in humans. To utilize RDA for this purpose, it is desirable to use pools of DNAs from a group of individuals for use as either tester, driver or both. When used this way, RDA may  
5 yield probes that detect polymorphic alleles that are present in one group and not in another. In particular, when such pools are used as driver, RDA yields probes for restriction endonuclease polymorphisms (PARFs) that distinguish tester from all individuals in the driver pool. When pools are used as  
10 tester, RDA yields PARFs that distinguish at least one member of the tester pool from the driver individual. In the most challenging example, when both tester and driver are pooled DNAs from groups of individuals, RDA yields PARFs that distinguish at least one member of the tester group from all  
15 members of the driver group.

This is illustrated in Table 4. Two groups of humans were taken: ten that shared a genetic abnormality, neuronal ceroid lipo-fuscinosis, also known as Batten's disease, and ten that did not have this condition. DNAs were prepared from cells of  
20 each individual and pooled accordingly. Pools of DNA were used for RDA using DNA from one group as tester and DNA from the other as driver, and then reversing the procedure. In each case difference products were obtained that detected PARFs. In Table 4 the probe name is listed, and "+" indicates that it  
25 detected the small allele of the PARF in a given individual. As the Table shows, when normal individuals were used as tester, probes (pA1, pA2, pA4, and pA9) were obtained that detected small PARF alleles in at least one member of the group, and this allele was always absent in the individuals  
30 with Batten's disease. Similarly, when DNAs from the affected group was used as tester, probes (pN2, pN7, pN9, pN13 and pN15) were obtained that detected small PARF alleles in at least one member of the affected group, and this allele was always absent in the normal group.

35 Table 4: Screening for presence of Bgl II PARF's in 20 human DNA amplicons

Length of

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Probe	Affecteds										Normals								small		allele (bp)
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	
5	pA1														+	+				+	300
	pA2													+	+			+	+	+	120
	pA4													+							150
	pA9														+	+				+	400
10	pN2										+										425
	pN7		+	+	+		+				+										300
	pN9										+										350
	pN13				+	+	+														400
	pN15										+										600

Example 7. The use of RDA in obtaining probes that reflect differences in RNA populations. RDA can be applied to compare populations of double stranded cDNAs derived from RNA. The difference products will yield probes that detect sequences expressed among the RNA from one source that are not equivalently expressed in another. Such probes are sometimes of use in diagnosis (e.g. to determine the origin of a cell, or to find evidence of infection) and can lead to the discovery of important tissue-specific or disease related genes.

A double stranded cDNA population was prepared from RNA extracted from a male mouse brain. This was used as driver. A one hundred thousandth part of double stranded DNA from the kanamycin resistance gene encoded by an E. coli plasmid was added to a small portion of this cDNA, and this used as tester. This model system mimics the case of a single small difference between the expressed RNAs from two sources. RDA was performed on these two samples using the enzyme Sau3A to prepare the respective amplicons. The difference product after two rounds of subtraction was separated using gel electrophoresis, as shown in Fig. 4. In the left hand lane is shown an electrophoretic separation of amplicons prepared from 1.2 kb of the kanamycin gene. In the middle lane were size markers. The difference product from the RDA is seen in the right hand lane. This product was derived from the kanamycin gene as shown by blot hybridization, thus proving that RDA can be used to detect differences in DNAs derived from RNA populations.

It is evident from the above results, that a powerful tool has been provided for isolating probes which can be used

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to identify sequence differences between two related genomes. This technique may be used in a wide variety of contexts in relation to forensic medicine, detecting the presence of pathogenic DNA, lesions occurring in neoplastic cells, genetic  
5 counseling, the presence of genes associated with genetic diseases, and the like.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically  
10 and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this  
15 invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A method for producing probes capable of distinguishing at least one sequence difference between DNA from two related different eukaryotic sources, said method  
5 comprising:
  - substantially completely digesting separately the DNA from said two different sources with a restriction endonuclease to provide digested fragments, wherein one of said sources is driver DNA, and the other source is tester DNA, wherein said  
10 tester DNA comprises target DNA, wherein said target DNA comprises sequence differences between the DNA of said two sources;
  - ligating a first set of adaptors to said digested fragments and amplifying said fragments using primers to one  
15 of the strands of said first set adaptors to provide amplified amounts of fragments of said digested sequences of less than about 2kbp as amplicons;
  - carrying out a first round of the following steps for enrichment of target DNA:
    - 20 removing said first set of adaptors from said amplicons and ligating a second set of adaptors to the 5' ends of the amplicons of tester DNA;
    - combining under melting and annealing conditions said tester amplicons with a large excess of driver amplicons,  
25 whereby a portion of the resulting dsDNA comprises self-annealed tester DNA including target DNA;
    - filling in the 3' ends of annealed DNA;
    - amplifying said dsDNA with primers complementary to one of said strands of said second set of adaptors to enrich for  
30 target DNA;
    - optionally repeating said first round of steps as a second round or successive round, to provide DNA sequences which serve to identify differences in DNA sequences between said tester source and said driver source.
- 35 2. A method according to Claim 1, including the additional step after said filling in of digesting single stranded DNA with a nuclease.

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3. A method according to Claim 1, wherein said first round of steps is repeated at least once.

4. A method according to Claim 3, wherein different sets of adaptors are used for at least the first three rounds.

5. A method according to Claim 1, wherein said digesting is with a restriction endonuclease which has a recognition sequence of at least 6 nucleotides and provides a staggered cleavage.

6. A method according to Claim 1, wherein the sources of DNA are cells from related human individuals or the same individual.

7. A method according to Claim 1, wherein said DNA from said two related sources is cDNA.

8. A method according to Claim 1, wherein said DNA from at least one of said two related sources is DNA pooled from a plurality of individual related sources.

9. A method for producing probes capable of distinguishing at least one sequence difference between genomes from two related cellular sources, said method comprising:  
substantially completely digesting separately the DNA from said two different sources with a restriction endonuclease having a nucleotide recognition sequence of at least 4 nucleotides, wherein one of said sources is driver DNA, and the other source is tester DNA, wherein said tester DNA comprises target DNA, wherein said target DNA comprises sequence differences between the genomes of said two sources;

ligating a first set of adaptors to said digested fragments and amplifying said fragments using primers to one of the strands of said first set adaptors to provide amplified amounts of fragments of said digested sequences of less than about 2kbp as amplicons;

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carrying out a first round of the following steps for enrichment of target DNA:

removing said first set of adaptors from said amplicons and ligating a second set of adaptors to the 5' end of  
5 amplicons of tester DNA;

combining under melting and annealing conditions said tester amplicons with a large excess of driver amplicons, whereby a portion of the resulting dsDNA comprises self-annealed tester DNA including target DNA;

10 filling in 3' overhangs;

amplifying said dsDNA with primers to one of said strands of said second set of adaptors to enrich for target DNA;

repeating said first round of steps for at least 2  
15 rounds, using a different set of adaptors in each successive round for said 2 rounds to provide a DNA composition comprising a predominant amount of target DNA;

cloning said DNA composition to provide clones having a substantially homogeneous probe of putative target DNA;

20 with the proviso that when a plurality of probes of putative target DNA are obtained, optionally including the additional step of:

hybridizing said probes of putative target DNA with driver and tester amplicons, whereby probes of putative target  
25 DNA binding to both driver and tester amplicons are discarded.

10. A method according to Claim 9, wherein said related human cellular sources are from the same individual and differ as to the suspected presence of a pathogen.

11. A method according to Claim 9, wherein said  
30 related human cellular sources are from the same individual and differ as to the suspected presence of a genetic lesion.

12. A method according to Claim 9, wherein said related human cellular sources are from different individuals.



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13. A method for producing probes capable of distinguishing at least one sequence difference between genomes from a neoplastic cell source and a related normal cell source, said method comprising:

5 substantially completely digesting separately the DNA from said two sources with a restriction endonuclease having a nucleotide recognition sequence of at least 4 nucleotides, wherein said normal cell source is driver DNA, and said neoplastic cell source is tester DNA, wherein said tester DNA  
10 comprises target DNA, wherein said target DNA comprises sequence differences between the genomes of said two sources comprising at least one of an insertion, deletion, rearrangement or DNA amplification defining target DNA;

ligating a first set of adaptors to said digested  
15 fragments and amplifying said fragments using primers to one of the strands of said first set of adaptors to provide amplified amounts of fragments of said digested sequences of less than about 2kbp as amplicons;

carrying out a first round of the following steps for  
20 enrichment of target DNA:

removing said first set of adaptors from said amplicons and ligating a second set of adaptors to 5' ends of amplicons of tester DNA;

combining under melting and annealing conditions said  
25 tester amplicons with a large excess of driver amplicons, whereby a portion of the resulting dsDNA comprises self-annealed tester DNA including target DNA;

filling in the 3' ends of overhangs;

amplifying said dsDNA with primers to one of said  
30 strands of said second set of adaptors to enrich for target DNA;

repeating said first round of steps for at least 1 additional round, using a different set of adaptors as to the previous round in each successive round to provide a DNA  
35 composition comprising a predominant amount of target DNA; and

cloning said DNA composition to provide clones having a substantially homogeneous probe of target DNA.

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14. A method for producing probes capable of distinguishing at least one sequence difference between genomes from a neoplastic cell source and a related normal cell source, said method comprising:

5 substantially completely digesting separately the DNA from said two sources with a restriction endonuclease having a nucleotide recognition sequence of at least 4 nucleotides, wherein said neoplastic cell source is driver DNA, and said normal cell source is tester DNA, wherein said tester DNA  
10 comprises target DNA, wherein said target DNA comprises sequence differences between the genomes of said two sources comprising loss of heterozygosity, homozygosity or hemizygous loss to define target DNA;

ligating a first set of adaptors to said digested  
15 fragments and amplifying said fragments using primers to one of the strands of said first set of adaptors to provide amplified amounts of fragments of said digested sequences of less than about 2kbp as amplicons;

carrying out a first round of the following steps for  
20 enrichment of target DNA:

removing said first set of adaptors from said amplicons and ligating a second set of adaptors to 5' ends of amplicons of tester DNA;

combining under melting and annealing conditions said  
25 tester amplicons with a large excess of driver amplicons, whereby a portion of the resulting dsDNA comprises self-annealed tester DNA including target DNA;

filling in the 3' ends of overhangs;

amplifying said dsDNA with primers to one of said  
30 strands of said second set of adaptors to enrich for target DNA;

repeating said first round of steps for at least 1 round, using a different set of adaptors as to the previous round in each successive round to provide a DNA composition  
35 comprising a predominant amount of target DNA; and

cloning said DNA composition to provide clones having a substantially homogeneous probe of target DNA.

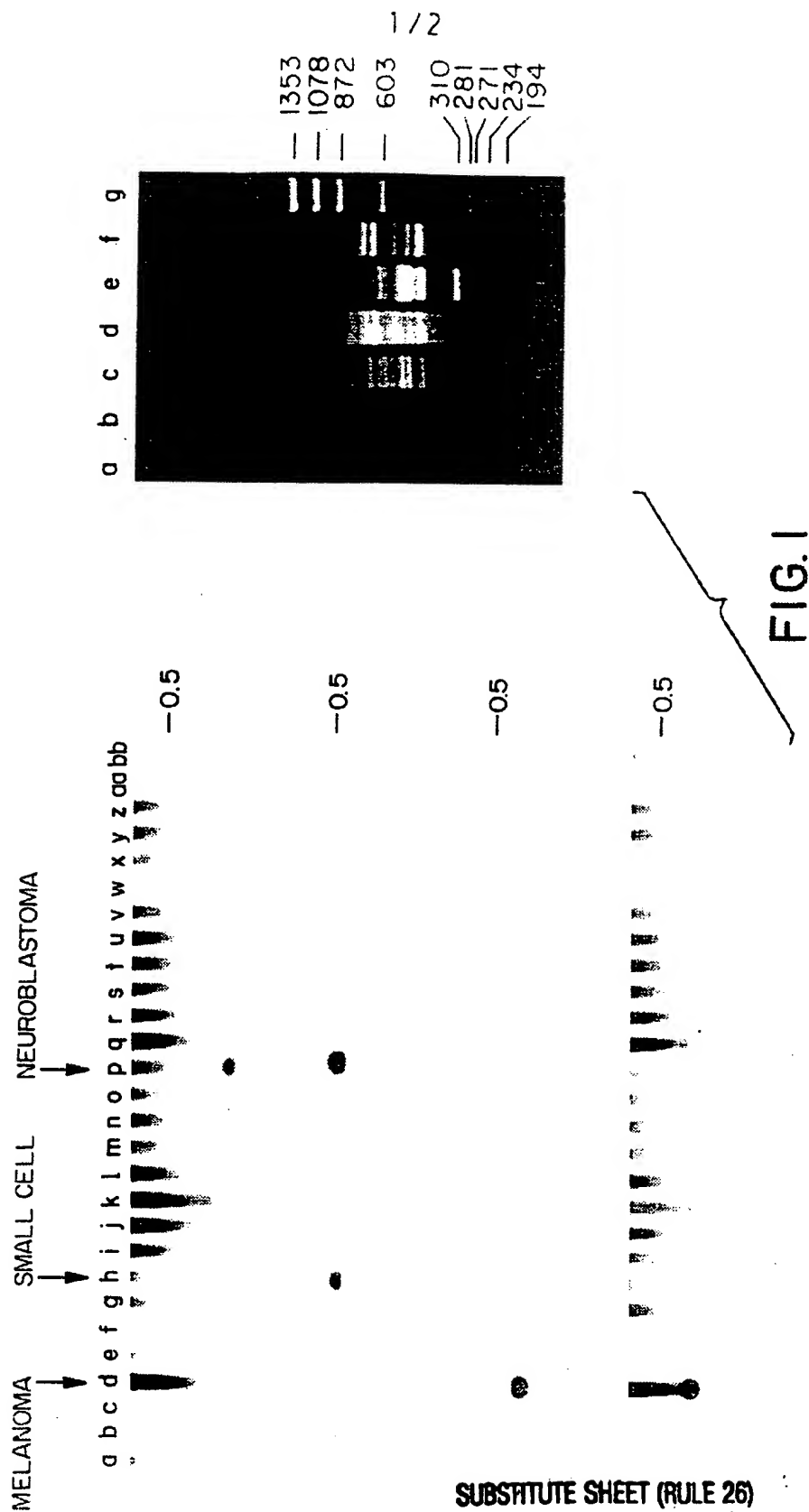
- 41 -

15. A kit comprising at least two probes prepared according to the method according to Claim 1.

16. A kit comprising at least two probes prepared according to the method according to Claim 9.

5 17. A kit comprising at least two probes prepared according to the method according to Claim 13.

18. A kit comprising at least two probes prepared according to the method according to Claim 14.



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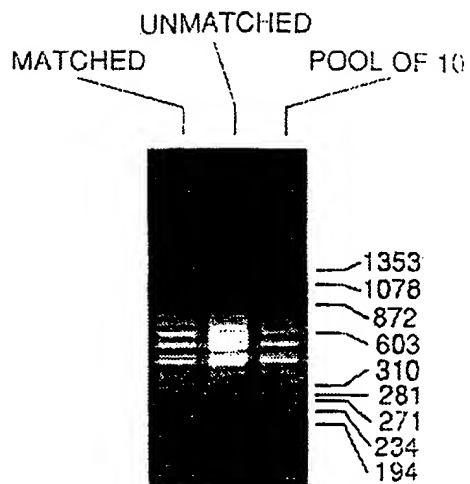


FIG. 2

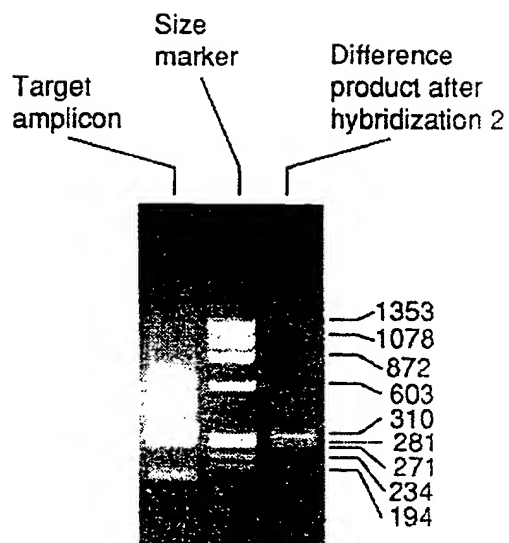


FIG. 4

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## RAT LINE SEQUENCE CLONED FROM HUMAN PROSTATE CANCER DNA SAMPLE

Human P35	438	GATCTTCCCGGTTGCTGCGCCACGAGAGACTTAGGCAGTATCCACGAGCAAACTTG	379
Rat L1Rn B6	1042	GATCTTCTGTTGCTGAGCGGAGAGAGCTCGAGGCGACACCCCGAGAGCGAACTTG	1101
Human P35	378	AGCCTTGGAAACACACAGGTAGGACCAACTTTTCCCTGCGAGNAAACCGCCTGGTGAATC	319
Rat L1Rn B6	1102	AGCCTCGGACACACAGGAGACCAACTTTTCTGCTGCAAGTGACCTGTCTGGTGAATC	1161
Human P35	318	AAGACACAGGCCACACAGAACAGCTGAAGACCTGTAGAGNGGAAAACTACATGCCCGAA	259
Rat L1Rn B6	1162	AAGACAGAGGCCACACAGAACAGCTGAAGACCTGTAGAGAGGAAAACTACACGCCCGAA	1221
Human P35	258	AGCAGAACACTCTGTCCCCCATTAAGTGGCTGAAAGAAACAGGAAACAGGTCTACAGCAC	199
Rat L1Rn B6	1222	AGCAGAACACTCTGTGCCCATTAAGTGGCTGAAAGAAACAGGAAACAGGTCTACAGCAC	1281
Human P35	198	TCTGACACACAGGCTTATAGGACAGTCTAGCCACGGTCAGAAATAGCAGAACAAAGTAA	139
Rat L1Rn B6	1282	TCTGACACACAGGCTTATAGGACAGTCTAGCCACGGTCAGAAATAGCAGAACAAAGTAA	1341
Human P35	138	CACTAGAGATAATCTGATGGCGAGAGGCGAGGAAACCCAGCAACAGAGACCAAGA	79
Rat L1Rn B6	1342	CACTAGAGATAATCTGATGGCGAGAGGCGAGGAAACCCAGCAACAGAGACCAAGA	1401
Human P35	78	CTACATGGCATCATCGGAGCCCAATTCTCCACCAAAATAAACATGGGATATCCAAACAC	19
Rat L1Rn B6	1402	CTACATGGCATCATCGGAGCCCAATTCTCCACCAAAATAAACATGGGATATCCAAACAC	1461
Human P35	18	ACCAGAAAAGCAACATC	2
Rat L1Rn B6	1462	ACCAGAAAAGCAAGATC	1478

FIG. 3

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/10722

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C07H 21/04; C12Q 1/68, 1/70; C12P 19/34  
US CL : 435/5, 6, 91.2; 536/24.3; 935/78, 80

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91.2; 536/24.3; 935/78, 80

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS

search terms: subtractive hybridization, difference analysis

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proc. Natl. Acad. Sci. USA, Vol. 87, issued April 1990, Wieland et al., "A method for difference cloning: gene amplification following subtractive hybridization", pages 2720-2724, see Fig. 1 on page 2721.	1-18
A	Proc. Natl. Acad. Sci. USA, Vol. 87, issued March 1990, Straus et al., "Genomic subtraction for cloning DNA corresponding to deletion mutations", pages 1889-1893, see Fig. 1 on page 1891.	1-18

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z	document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means		
*P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

31 January 1994

Date of mailing of the international search report

15 FEB 1994

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/10722

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nucleic Acids Res., Vol. 19, Number 25, issued 1991, Hara et al., "Subtractive cDNA cloning using oligo(dT)30-latex and PCR: isolation of cDNA clones specific to undifferentiated human embryonal carcinoma cells", pages 7097-7104, see Fig. 2 on page 7100.	1-18
A	Biochem. Biophys. Res. Comm., Vol. 167, Number 2, issued 16 March 1990, Hla et al., "Isolation of immediate-early differentiation mRNAs by enzymatic amplification of subtracted cDNA from human endothelial cells", pages 637-643, see Fig. 1 on page 640.	1-18